

University of Zagreb
Faculty of Science
Department of Biology

Duje Burić

**Characterization of ubiquitin-binding domain in C1orf124
protein**

Graduation thesis

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This diploma thesis was made at the Mediterranean Institute for Life Sciences (MedILS), Split, Croatia, in the Tumor Biology Program and at the Institute of Biochemistry II, Goethe University School of Medicine, Frankfurt, Germany, in the Molecular Signalling Group under supervision of Dr. Ivan Đikić.

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CHARACTERIZATION OF UBIQUITIN BINDING DOMAIN IN C1orf124
PROTEIN

Duje Burić

Mediterranean Institute for Life Sciences (MedILS), Split, Croatia

Summary

Ubiquitin has recently been discovered to have a key role in a variety of biological processes. In parallel, different protein domains were identified with the ability to bind different ubiquitin species. Ubiquitin-binding zinc-finger 4 (UBZ4) family was recently revealed using bioinformatical analysis. UBZ4 is a C2HC Zn-finger found in all eukaryotic lineages; most of the UBZ4-containing proteins appear to play a role in the DNA damage response.

Iterative alignment with known UBZ4 revealed 13 members of putative family. C1orf124 protein was chosen to analyse its ubiquitin binding abilities. I have showed that C1orf124 protein binds monoubiquitin and polyubiquitin chains. Pull down assay showed that interaction is achieved through hydrophobic patch surrounding Ile44 on ubiquitin and Asp residue in-between second Zn-binding dyad of UBZ4 domain.

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Co-supervisor: Asst. Prof. Maja Matilić, PhD

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KARAKTERIZACIJA DOMENE KOJA VEZUJE UBIKVITIN U PROTEINU

C1orf124

Duje Burić

Mediterranski institut za istraživanje života (MedILS), Split, Hrvatska

Sažetak

Novija istraživanja pokazala su da ubikvitin ima ključnu ulogu u različitim biološkim procesima. Također su otkrivene proteinske domene koje mogu vezivati različite ubikvitinske vrste. Bioinformatička analiza nedavno je otkrila obitelj domena koje vezuju ubikvitin sa specifičnom strukturom cinkovog prsta, tzv. domenu UBZ4. Sadrži cinkov prst C2HC i nalazimo je u proteinima eukariota svih linija. Za većinu proteina koji sadrže UBZ4 domenu vjeruje se da imaju bitnu ulogu u odgovoru na oštećenja DNA molecule u stanici.

Iterativno sravnjivanje s poznatim UBZ4 otkrilo je 13 članova pretpostavljene obitelji. Protein C1orf124 je izabran za istraživanje njegovog vezanja na ubikvitin. Ovim istraživanjem pokazao sam da protein C1orf124 veže monoubikvitin i poliubikvitinske lance. Afinitetna kromatografija pokazala je da se interakcija postiže preko hidrofobne regije na površini ubikvitina koja vrlo konzerviran izoleucinski aminokiselinski ostatak na 44 mjestu i aspartatnog ostatka unutar druge dijade koja vezuje atom cinka domene UBZ4.

(61 stranica, 11 slika, 10 tablica, 71 literaturnih navoda, jezik izvornika: engleski)

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Voditelj: Dr. sc. Ivan Đikić, prof.

Suvoditelj: Dr. sc. Maja Matulić, doc.

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Abbreviations

CUE – Coupling of Ubiquitin conjugation to Endoplasmic reticulum degradation
DUB – Deubiquitinating Enzyme
DUIM – Double-sided UIM
GAT – GGA and TOM
GGA – Golgi-localized, gamma-ear-containing, ADP-ribosylation-factor-binding protein
GLUE – GRAM-like ubiquitin-binding in Eap 45
Ile – Isoleucine
Lys – Lysine
LZ – Leucine zipper
MIU – Motif interacting with ubiquitin
NZF – Npl4 zinc finger
PAZ – Polyubiquitin-associated zinc binding
PFA – Paraformaldehyde
Ub – Ubiquitin
UBA – Ubiquitin associated domain
Ubc – Ubiquitin conjugating enzyme
UBD – Ubiquitin binding domain
UBZ – Ubiquitin-binding zinc-finger
UEV – Ubiquitin-conjugating enzyme variant
UIM – Ubiquitin interacting motif
TCL – Total cell lysate
TOM – Target of Myb

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1. Introduction

1.1. Ubiquitination

Ubiquitin (Ub) is ubiquitously expressed 76-amino-acid long polypeptide. We can find it in a huge number of species starting from yeast to human, and it is highly conserved protein. It is involved in a process called ubiquitilation, which is a covalent modification of protein. Originally described as destruction tag for misfolded or disused proteins Ub has recently been discovered as a key player in variety of other fundamental processes such as DNA repair, transcriptional regulation, signal transduction, cell cycle control and vesicular traffic. Similar to phoshporylation, ubiquitination is an inducible and reversible process that changes the properties of the modified substrate; for example, its subcellular localization, stability or enzymatic activity. In these processes, protein ubiquitination exhibits inducibility, reversibility and recognition by specialized domains, features similar to protein phosphorylation, which enable ubiquitin to act as a signalling device.

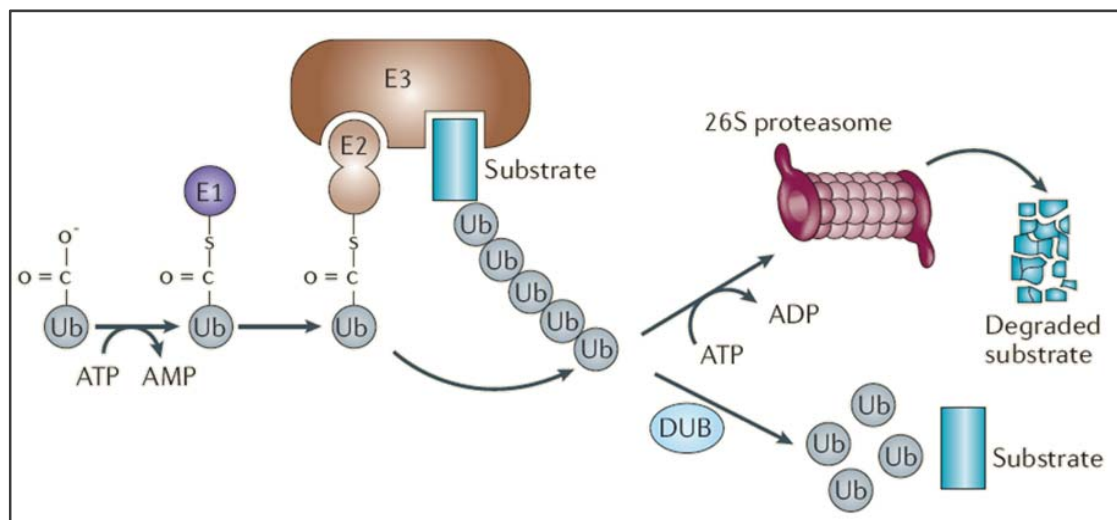


Figure 1. ATP-dependent activation of Ub. Ub is activated in an ATP-dependent manner by an ubiquitin-activating enzyme (E1), and is then transferred to a ubiquitin-conjugating enzyme (E2) through thioester bond. An Ubiquitin-protein ligase (E3) specifically attaches ubiquitin to the ϵ -amino group of a lysine residue in the target protein. Iterative addition of new Ub moieties to the Lys48 residues of conjugated Ub leads to polyUb chain formation. The substrate protein is then recognized and targeted for proteasomal degradation. Ubiquitination is a reversible process, in which Ub moieties are removed through the action of deubiquitinating enzymes (DUBs). (Source: Hoeller *et al.*, 2006)

In this post-translational protein modification Ub is covalently attached to target protein via an isopeptide bond between the carboxyl-terminal glycine (Gly-76) of Ub

and the ϵ -amino group of lysine of substrate proteins. The modification occurs in a three step enzymatic process and results in attachment of monoUb or polyUb chains to proteins: 1) The C-terminus of Ub is activated in an ATP-dependent manner by forming a thiol ester with cysteine residue of ubiquitin-activating enzyme (E1); 2) Ub is transferred to the active site cysteine of conjugating the enzyme (E2); and 3) single or multiple Ubs are transferred from E2 to the lysine residue of the target protein in reactions catalyzed by the ubiquitin ligase (E3) (Figure 1) (Hershko and Ciechanover, 1998).

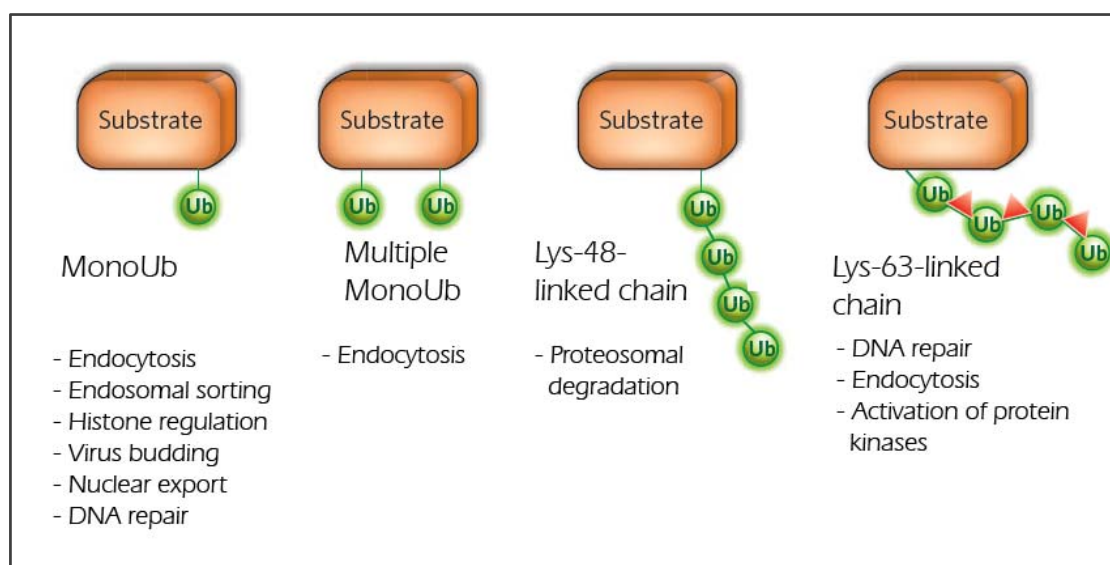


Figure 2. Types of ubiquitination: Several types of ubiquitination are correlated with regulation of different cellular processes. **a)** The simplest one is monoubiquitination and it regulates endocytosis, endosomal sorting, DNA repair and many others. **b)** Addition of several single Ub molecules to different Lys residues results to multiple monoubiquitination and this modification is implicated in endocytosis. Polyubiquitination results from the attachment of a chain of Ub molecules to one or more Lys residues. **c)** Ub chains formed via Lys48 are targets for proteosomal degradation whereas **d)** chains linked via Lys63 are enrolled in DNA repair, endocytosis and activation of protein kinases. (Source: Hoeller *et al.*, 2006)

There are several types of Ub modifications. The simplest type is defined as monoubiquitination where a single Ub moiety is attached (Hicke and Dunn, 2003). Alternatively, the substrate can be tagged with single Ub molecules on several lysine residues, giving rise to multiple monoubiquitination, known as multiubiquitination (Haglund *et al.*, 2003). Polyubiquitination is also possible, because Ub contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) which can be targeted by other Ubs in an iterative process. This process leads to the formation of an

Ub chain which is attached to a single lysine residue on substrate (Pickart and Fushman, 2004).

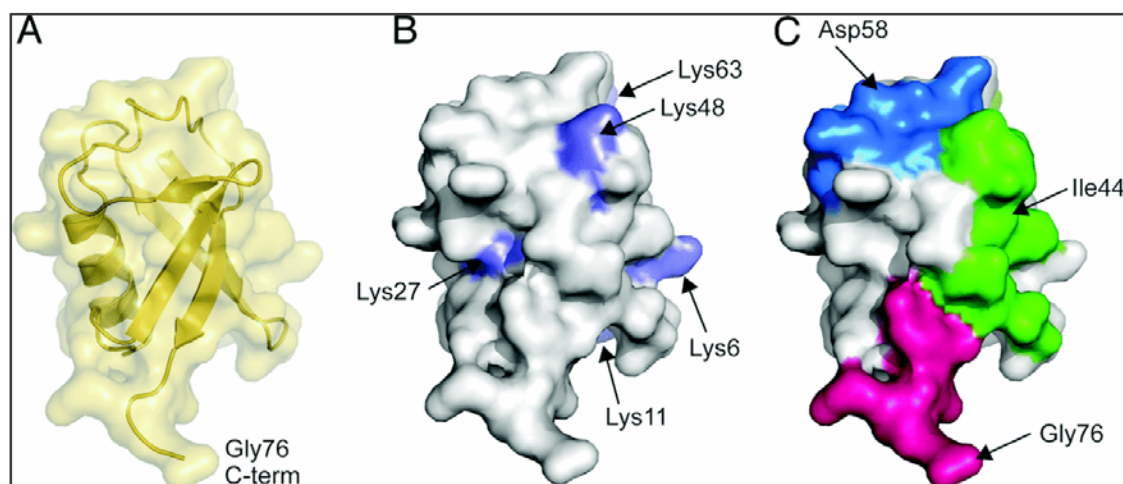


Figure 3. Structural features of Ub. A) Ribbon and surface representations of Ub. The C terminal Gly76 through which Ub can bind its target proteins is marked. B) Lysine residues in Ub (blue) which can covalently bind other Ubs. C) Major recognition patches on Ub. The hydrophobic patch centred on Ile44 (green), the polar patch centred on Asp58 (blue) and the diglycine patch near the C-terminal Gly76 (pink) are shown (Source: Hurley *et al.*, 2006).

Different types of ubiquitination conjugates are engaged in regulation of different kinds of cellular processes. It is clear that polyUb chain formed through Lys48 has a role in targeting proteins for 26S proteasomal degradation, whereas Ub chains formed via Lys63 are involved in processes of endocytosis and DNA repair (Hershko and Ciechanover, 1998; Hofmann and Pickart, 2001). Monoubiquitination is an important signal during receptor endocytosis. It functions as an endosomal sorting signal targeting cell surface receptors for lysosomal degradation. Similarly, monoUb attached to biosynthetic and endocytic membrane proteins is a signal for cargo sorting into vesicles that bud into the late endosome lumen for delivery into the lysosome and it is implicated in DNA repair, histone activity and transcriptional regulation (Figure 2.) (Dunn and Hicke, 2003).

Non-proteasomal, Ub signals are based on monoubiquitination or other types of polyUb chains, including those linked through Lys63 and Lys6 (Ikeda and Dikic, 2008; Hoffman, 2009). Over the last years a number of proteins were found to be ubiquitinated upon irradiation or treatment with DNA-damaging agents such as PCNA (Hoege *et al.*, 2002), the core histone H2A and its variant H2AX (Bergink *et*

al., 2006), the 9-1-1 complex (Fu *et al.*, 2008), the Fanconi pathway proteins FANCD2 and FANCD1 (Smogorzewska *et al.*, 2007), and the replication factor Rfc2 (Tomida *et al.*, 2008).

The Ub marks at the damage site are based on monoUb or Lys63-chains. The downstream proteins have to recognize these modifications in a background of different constitutively ubiquitinated proteins.

The human genome encodes about 40 different E2 enzymes and more than 500 different E3 ligases, most of which are probably actively involved in protein ubiquitination.

1.2. Ubiquitin as an inducible and reversible signal

It is well known that protein ubiquitination is induced by a variety of stimuli. For instance, many cell surface receptors become ubiquitinated upon extracellular ligand stimulation (Dunn and Hicke, 2003). In addition, many cytoplasmic and nuclear proteins become ubiquitinated following their phosphorylation (Di Fiore *et al.*, 2003). Ubiquitination shares many similarities with protein phosphorylation. The signal-inducible substrate recognition and substrate specificity enabled by Ub ligases are very important. The functions of Ub ligases are tightly regulated by mechanisms such as compartmentalization, degradation, oligomerization and post-translational modifications (Dikic *et al.*, 2003). E3 ligases play an important role in ubiquitination process, because they recognize the acceptor protein and for that reason they dictate the specificity of the reaction. There is a huge number of different Ub ligases present in cell emphasizing the need for their controlled regulation.

The second key feature is deubiquitination, Ub removing mediated by specific enzymes (DUBs). These enzymes are responsible for switching off the Ub signal or shifting between different modifications of the same Lys residue (Hershko and Ciechanover, 1998). Both of modifications are recognized by specific protein domains, providing a mechanism for translation of the Ub or phospho-specific signal to downstream effectors (Hicke *et al.*, 2005). Phosphorylation and ubiquitination are in tight connection in the cells and usually phosphorylation is a signal preceding ubiquitination. The main differences between these two signaling systems are that Ub is chemically more complex than phosphate and forms chains of different conformations, indicating distinct targets and functions in the cell (Pickart, 2000).

The Ub modifications affect the ability of target protein to interact with other and this is one of the keys to understand how Ub is involved in such a variety of cellular processes. This regulation necessitates the existence of interactors with distinct binding specificities and effectors functions. Consequently, a growing number of Ub-interacting proteins with many specialized Ub-binding domains (UBDs) combined with a variable effector domains have been identified (Hicke *et al.*, 2005; Hurley *et al.*, 2006). Other proteins of Ub-related processes have evolved domains with Ub-like structures that share structural similarity with Ub (Ub fold) and mimic certain aspects of ubiquitination. They are known as Ub-like modifiers. Hence, they are conjugated to proteins and function in an "ubiquitin-like" manner (Welchman *et al.*, 2005).

1.3. Ubiquitin binding domains

Typical Ub-binding domains have been initially discovered in bioinformatical sequence database searches. They appear as regions of locally confined sequence similarity shared by multiple proteins known or suspected to bind to Ub (Hoffman, 2005). Like other functional protein domains, the dedicated UBDs can fold independently of the rest of the host protein, and can – at least to a certain degree – also function in isolation. For most of the predicted UBD classes, experiments have demonstrated that a majority of proteins harboring these domains actually do bind to Ub, although the affinities and chain preferences may vary considerably (Varadan *et al.*, 2005)

Usually, structures of isolated UBDs are not very informative, as UBDs are rather small modules of 15–60 residues and assume simple folds. More useful are structures of Ub–UBD complexes, which reveal the binding mode and the molecular surfaces involved. With very few exceptions, the truly modular UBDs exhibit two interesting trends in Ub recognition: (i) the UBD surface in contact with Ub is typically contributed by an α -helix of the UBD, and (ii) the Ub surface recognized by the UBD typically includes a hydrophobic surface patch surrounding the highly conserved Ile-44 residue of Ub.

Ub-binding domains can be classified into a number of different families, whose members share sequence and structural similarity only within the family. Currently, more than ten such families are known, each of them with multiple members in a given genome.

Table 1. Complex structures and binding affinities of ubiquitin binding domains.

Ub-binding domain	Source protein	Binding affinity Kd (μ M)	Selected references
UBA	Dsk2	14.8 ± 5.3	Ohno <i>et al.</i> , 2005
	hHR23A	400 ± 100 (monoUb)	Varadan <i>et al.</i> , 2005
	Mud1	390 ± 50 (monoUb)	Trempe <i>et al.</i> , 2005
	Ede1	83 ± 9	Swanson <i>et al.</i> , 2006
CUE	Vps9	20 ± 1	Prag <i>et al.</i> , 2003
	Cue2	155 ± 9	Kang <i>et al.</i> , 2003
GAT	GGA3	181 ± 39	Prag <i>et al.</i> , 2005
	GGA3		Kawasaki <i>et al.</i> , 2005
	TOM1	409 ± 13	Akutsu <i>et al.</i> , 2005
UEV	Vps23		Teo <i>et al.</i> , 2004
	Tsg101	510 ± 35	Garrus <i>et al.</i> , 2001
Ubc	UbcH5	~ 300	Brzovic <i>et al.</i> , 2006
UIM	Vps27	277 ± 8 (UIM1)	Swanson <i>et al.</i> , 2006
		177 ± 17 (UIM2)	
DUIM	Hrs	190 (wt)	Hirano <i>et al.</i> , 2006
MUI	Rabex-5	29 ± 4.8	Lee <i>et al.</i> , 2006
PAZ	mHDAC6	~ 58	Seigneurin-Berny <i>et al.</i> , 2001
NZF	Npl4	126 ± 26	Alam <i>et al.</i> , 2004
GLUE	Eap45	~ 135	Slagsvold <i>et al.</i> , 2005
A20 ZnF	Rabex-5	22 ± 0.4	Lee <i>et al.</i> , 2006
ZnF UBP	Isopeptidase 5	2.8	Reyes-Turan <i>et al.</i> , 2006
VHS	Vps27	150	Mizuno <i>et al.</i> , 2003
	HRS		
	STAM		

The first Ub-binding site to be characterized was found in a proteasome subunit present in proteasome subset, the S5A/RPN10 protein¹¹. The S5a sequence,

necessary and sufficient for interactions with Ub, is short and simple, and was used as a starting point in several bioinformatics searches to identify similar sequences in other proteins. Hidden Markov models and iterative database searches that were based on the S5a sequence identified a sequence pattern known as the ubiquitin-interacting motif (UIM) (Hoffman and Falquet, 2001; Donaldson *et al.*, 2003). Like the original S5a UIM, UIMs in a number of diverse proteins were quickly shown to be direct, bona fide ubiquitin-binding motifs.

The UIM consists of a single α -helix, surrounded by a conserved alanine residue. The UIM helix enters in a shallow hydrophobic groove on the surface of Ub, and the alanine residue interacts with Ub Ile44. Other interactions are centred around Ile44 and cover a modest amount of surface area, consistent with the low affinity interactions (Fisher *et al.*, 2003; Swanson *et al.*, 2003).

Two recently described UIM variants illustrate the versatility of single helix-based Ub recognition. The MIU is a single helix that, so far, seems to be unique to one protein, the Rab5 exchange factor Rabex-5 (Lee *et al.*, 2006; Penengo *et al.*, 2006). The MIU is centered on a functionally essential alanine residue that contacts Ub Ile44. The MIU helix sits in the same hydrophobic groove that binds the UIM, but does so in the opposite orientation. The MIU is a remarkably clear-cut and elegant example of convergent evolution.

The DUIM is another remarkable variation on the UIM theme. One face of the conventional UIM helix binds Ub, whereas the other face is exposed to solvent. In the DUIM, two UIM sequences are interlaid on a single helix such that both faces are capable of binding Ub (Hirano *et al.*, 2006). The DUIM provides a mechanism for binding two, rather than one, Ub moiety, which provides an alternative to a double repeat of a conventional UIM.

Another motif, the ubiquitin-associated (UBA) domain, was the first identified using bioinformatics techniques as a sequence pattern common to a subset of proteins that are involved in ubiquitination or deubiquitination reactions (Hoffman and Bucher, 1996). UBA domains are compact three-helix bundles (Davies *et al.*, 2004; Miller *et al.*, 2004). PolyUb binding is the most established physiological function for the UBA domain (Tanaka *et al.*, 2003; Raasi *et al.*, 2004).

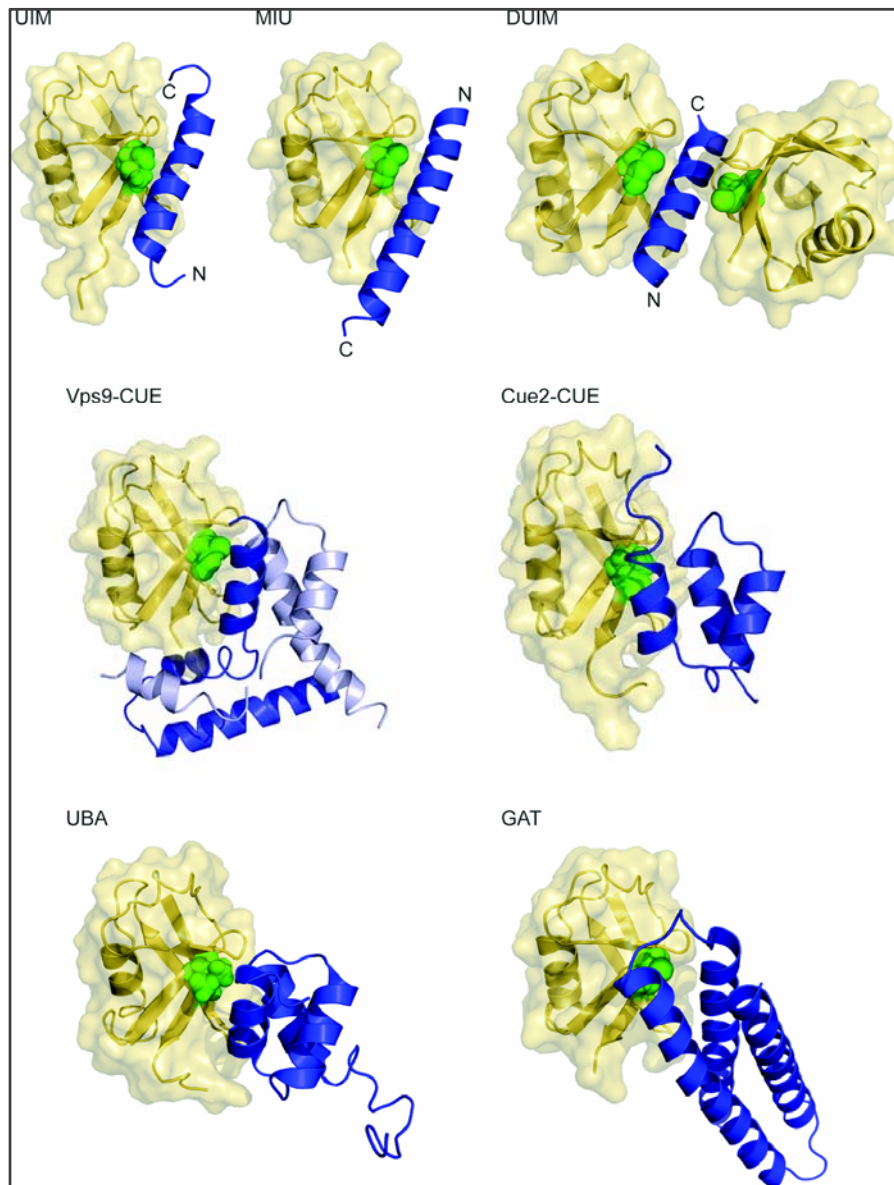


Figure 4. Helical ubiquitin-binding domain structures. Ub molecule (yellow) in ribbon and surface representations is shown with corresponding helical domain (blue) in ribbon representation. Ile44, the centre of the hydrophobic recognition patch on the Ub, is shown as green spheres. (Source: Hurley *et al.*, 2006)

UBA domains bind monoUb *in vitro* (Kato *et al.*, 2004; Kang *et al.*, 2003) and have been found to play a role in a variety of other protein–protein interactions. The Ile44 patch on monoUb binds to a conserved hydrophobic patch on the $\alpha 1$ and $\alpha 3$ helices of the UBA domain.

The largest class of ubiquitin-binding domains are α -helical: UBA (ubiquitin associated), UIM (ubiquitin-interacting motif), DUIM (double-sided UIM), MIU (motif interacting with Ub) and CUE (coupling of Ub conjugation to endoplasmic reticulum degradation). All of the helical ubiquitin-binding domains are known to

interact with a single region on Ub, the Ile44 hydrophobic patch. The UBA and CUE domains have structural homology, with common three-helical bundle architecture. They also have similar modes of binding to the Ile44 patch. The UIM and GAT domain structures are unrelated, except for being helical, and they interact with this patch in different ways. One of them is octahelical VHS (Vps27 (vacuolar protein sorting)/Hrs/STAM) domain (Hurley *et al.* 2006). The other two are GAT (Gga and TOM1) and PAZ (polyubiquitin-associated Zinc finger) UBDs, found in two-hybrid screens that used bait proteins not previously known to bind ubiquitin (Yamakami *et al.*, 2003; Scott *et al.*, 2004).

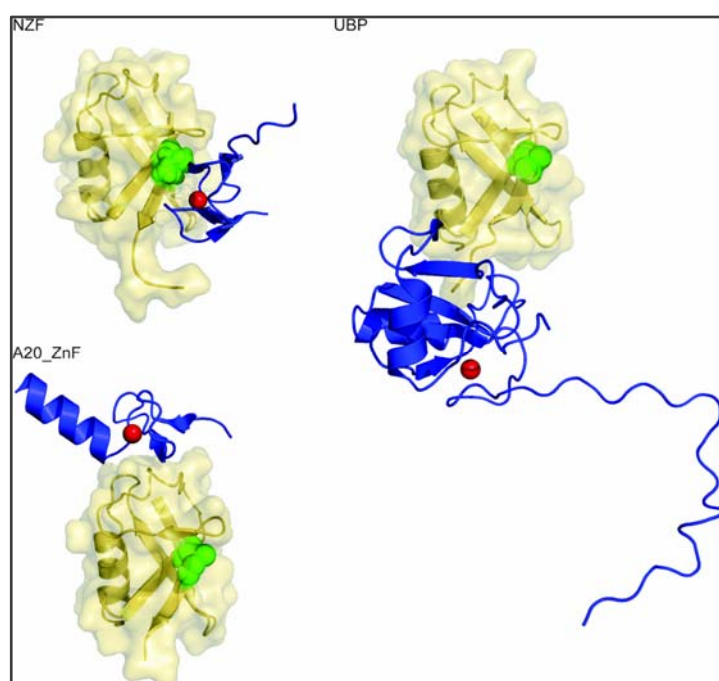


Figure 5. ZnF domain structures. Three ZnF domains (NZF, UBP and A20 ZnF) are shown (blue) in ribbon representation, with Ub (yellow) in ribbon and surface representations. Ile44, the centre of the hydrophobic recognition patch on the Ub, is shown as green spheres. (Source: Hurley *et al.*, 2006)

The PAZ domain was also discovered to bind Ub in biochemical experiments (Segneurin-Berny *et al.*, 2001), as was another type of zinc finger ubiquitin-binding motif, the NZF (Npl4 zinc finger) motif (Meyer *et al.*, 2002; Kanayama *et al.*, 2004), as well as the VHS (Vps27, HRS, STAM) (Mizuno *et al.*, 2003) and GLUE (GRAM-like ubiquitin-binding in Eap45) (Slagsvol *et al.*, 2005) domains. At the end of the list is the UEV (ubiquitin-conjugating enzyme variant) motif, a domain similar to

catalytic domain of E2s (ubiquitin-conjugating enzymes) but without the active-site cysteine. Despite the structural relationship between the UEV and E2 catalytic domains, UEV domains are non-catalytic and function as non-covalent ubiquitin-binding sites in proteins with disparate functions.

There is a wide range in UBD – Ub affinities, but these interactions — especially those with monoUb — are on the low-affinity end of the scale (they typically have a K_d of 10–500 M). Biologically relevant, low-affinity protein–protein interactions are not without precedent. Weak UBD – Ub interactions are probably physiologically relevant because point mutations could be detrimental *in vivo* (Alam *et al.*, 2004; Shih *et al.*, 2002). UBD – interactions might be relatively weak because they function in reversible, transitory protein networks similar to one described above. Examples are UBD – Ub interactions that are probably part of the network required for the plasma membrane vesicles budding, because numerous endocytic proteins have UBDs and/or are monoubiquitinated (Hicke and Dunn, 2003)). In these cases, the modification of a protein with Ub would function as a switch recognized by UBDs that controls the regulated assembly of a network, as has been proposed for Src-homology-2 (SH2)-DOMAIN–phosphotyrosine interactions and other regulatory switches (Lim *et al.*, 2002). Thanks to the presence of many deubiquitinating enzymes (DUBs) in most cells, ubiquitin-induced switches can be quickly reversed and individually regulated.

Another reason for low-affinity UBD – Ub interactions might be the relatively high concentration of the free Ub in the cells (estimated to be 10 M in mammalian cells (Haas and Bright, 1985). An exposed UBD would be constitutively occupied with free Ub and unavailable for binding to a ubiquitinated partner. So, for higher affinity interactions the strength is achieved by the presence of several UBD motifs in the receptor or receptor complex, by the multimerization of Ub receptors, or by further contacts between the Ub receptor and the ubiquitinated target.

The regulation of Ub binding domain can be carried through several mechanisms. One way is the regulation of the UBD accessibility. Several UBDs bind to Ub more effectively when they are outside the context of the full-length protein (Seigneurin-Berny *et al.*, 2001). This indicates that interactions between Ub and UBDs are controlled by inter- or intramolecular interactions, or by post-translational modifications. UBD accessibility might be controlled by steric occlusion: intramolecular interaction between a UBA domain and a Ubl domain inhibits one Ub receptor that shuttles proteins to the proteasome, RAD2 (Walters *et al.*, 2003). It is

clear that participation in other intra- or intermolecular protein–protein interactions is a mechanism that regulates the ubiquitin-binding ability of some UBDs. It is possible that other mechanisms of UBD regulation exist, such as post-translational modification and the control of subcellular localization.

1.4. Ubiquitin binding zinc finger families

One of the newest discovered Ub binding domain families is the ‘ubiquitin-binding Zn-finger’ (UBZ) family. It has been discovered through bioinformatical analysis of several yeast two-hybrid screens aimed at finding unconventional Ub interactors (Bienko *et al.*, 2005).

Ubiquitin-binding Zn-finger family is a group of proteins detected by the two-hybrid screen and characterized by short mono-nucleate Zn-fingers within their minimal interaction region. Among the proteins identified was TAX1BP1 with two copies of a C2H2-finger (Iha *et al.*, 2008) and the uncharacterized protein FLJ44922 with one copy of a C2HC-finger. Both Zn-finger types were distantly related to each other, but also to a large class of DNA-binding Zn-fingers. Due to their experimentally confirmed binding to Ub (Bienko *et al.*, 2005; Iha *et al.*, 2008), they are referred to as ‘ubiquitin-binding Zn-fingers’ UBZ1 (TAX1BP1-family) and UBZ2 (FLJ44922 family). A bioinformatical search for Zn-fingers with more similarity to UBZ1/2 than to the DNA-binding Zn-fingers revealed a number of additional UBZ candidate families, UBZ3 up to UBZ9 (Koraljka Husnjak and Ivan Dikic, unpublished results). Of particular interest are the UBZ3 and UBZ4 families, as they are highly enriched in DNA damage response proteins, and their ubiquitin-binding properties have been firmly established (Bienko *et al.*, 2005; Bish and Myers, 2007; Crosetto *et al.*, 2008)

UBZ3, UBZ4

hPol η		QVPC CE K CG SLVPVWD...MP EH MDY HFA LEL
dmPol η	1	T TK CD Q CG ANIPDEVK.S LQ TRD HFA QEL
	2	M NQ CE P CK AFIKCVD...MP EH L DYH VAKNL
spPol η		TYT CEE CE QKITLSE...R NEH EDY HT ALS I
yRad30		K TE CE CKYQVTFTDQKA...L QEH ADY H AL KL K
struct		eee eeee hhhhhhhhhhhhhh

hWRNIP1		QV CP V CG Q MM PA AI ...N SH LDR CL LL HP
yMgs1		L TS CP I CS E KV FFSLI...N SH L DI CG KEKS
hRad18		K VD CP V CG VN IP ESH...N KL LDS CL SREE
yRad18		MA Q CP I CG QFYPLKALE...K TH L DE CL TLQS
hArtemis		DGY CP N CG MPFSS LI QTPRW HV F EC LDSPP
yPso2		V TC CP I CL ENL SH LE Y ER TH CD TC IGSDP
hPol κ	1	L IT CP V CF RA QG CT SLE ALN KH VD EC LDGFS
	2	A LV CP CLN VE QK TS DL TL FN VH VD CL NK SF
hRAP80	1	Q VS CP CL CD QC FP TK I...E RH AM YCN GLME
	2	NE KCY L CK SLVP PF REY...Q CH VD SC LQ LAK

Figure 6. Multiple alignment of different members of UBZ 3 and UBZ4 family. Multiple alignment of human and yeast members of the UBZ3 and UBZ4 families (Zn-coordinating residues in yellow). Residues involved in ubiquitin-binding are labeled by blue asterisks. (Source: Hofmann, 2009)

The UBZ3 family is a C2H2 Zn-finger and has only one family member, the translesion DNA polymerase η (corresponding to Rad30 of budding yeast). Mammalian and fungal versions of this Y-family polymerase have a single copy of the UBZ3 finger, while the insect version contains two copies in the C-terminal region. The ubiquitin-binding properties and function of the UBZ3-containing Pol η are similar to that of the UBM-containing polymerases Pol ι and Rev1 (Bienko *et al.*, 2005). An NMR structure of the UBZ3 domain of Pol η (Bomar *et al.*, 2007) shows that the ubiquitin-interacting surface resides on a helix surface pointing away from the Zn-ligand.

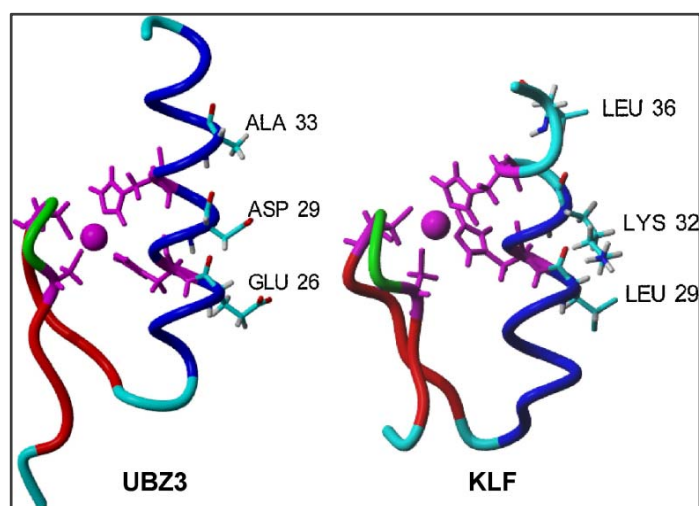


Figure 7. Comparison of a UBZ structure with a DNA-binding Zn-finger. In the UBZ3 structure (left, PDB: 1I5O); Zn and Zn-binding residues are shown in purple. Three residues in contact with Ub are shown as sticks. The DNA-binding KLF finger (right, PDB: 1P7A) is shown for comparison in the same orientation. The three residues shown as colored sticks correspond to the ubiquitin-binding residues of the UBZ3 finger. (Source: Hofmann, 2009)

The UBZ4 family of domains is a C2HC Zn-finger found in several proteins from all eukaryotic lineages; most of the UBZ4-containing proteins appear to play a role in the DNA damage response. So far, ubiquitin-binding has been demonstrated for three UBZ4 proteins: the Y-family translesion polymerase κ (Bienko *et al.*, 2005), the Werner-helicase interacting protein WRNIP1 (Bish and Myers, 2007; Crosetto *et al.*, 2008) and the ubiquitin ligase Rad18 (Notenboom *et al.*, 2007). No ubiquitin-binding has been tested for other UBZ4 proteins involved in the DNA damage response, such as Artemis/Pso2, and RAP80. Structural information on the UBZ4 domain is currently not available, although it is predicted to assume a fold analogous to UBZ3. However, the three UBZ3 residues in direct contact with Ub (Bomar *et al.*, 2007) are not very well conserved in UBZ4, and even less so in the UBZ1 and UBZ2 families.

1.5. Wrnip1 is a member of UBZ4 family

Wrnip1 (Werner helicase-interacting protein 1) is a protein with UBZ4 domain that can form homo-octameric complex (Tsurimoto *et al.*, 2005). It has been implicated in

the bypass of stalled replication forks in bakers' yeast. However, the function(s) of human Wrip1 has remained elusive so far. Recent experiments showed that Wrip1 was able to bind monoUb as well as polyUb chains (Crosetto *et al.*, 2008). It has been described as a novel modulator for initiation or restart events during pol γ -mediated DNA synthesis. Its ATP-ase activity is utilized to sense the DNA ends and to regulate the extent of stimulation (Tsurimoto *et al.*, 2005). In the nucleus of cultured cells human Wrip1 is concentrated in a variety of structures, most of which have a punctuated, focal appearance and are visible throughout the cell cycle. A number of these foci overlap with replication factories, and the presence of Wrip1 at DNA replication sites is greatly increased upon stalled replication forks, such as after UVC. The presence of Wrip1 at sites other than replication foci also hints at possible functions beyond DNA replication.

Recent findings showed that for localization of Wrip1 inside replication factories UBZ domain is indispensable and is significantly enhanced by UVC irradiation. On the other hand, UBZ domain is not responsible for Wrip1 homo-oligomerisation. The formation of oligomers is important for its presence inside nuclear foci. It has been shown that lacking of predicted leucine zipper (LZ) at position 496-547 (Kawabe *et al.*, 2006) severely affected the ability of Wrip1 to oligomerise.

ATP-ase activity has also been shown present in Wrip1. It can be stimulated by specific DNA structures such as DNA termini (Tsurimoto *et al.*, 2005). This function clearly suggests that Wrip1 could be a chaperone engaged in several transactions in the nucleus (Crosetto *et al.*, 2008).

Wrip1 is a new member of a growing family of UBD-containing proteins that use their specific UBDs to localize in nuclear focal structures, not only after induced DNA damage but also in unstimulated cells. Its UBZ4 domain is clearly responsible for its engagement in DNA repair processes. This is the confirmation that this module of UBZ domain can indicate the relationship between the protein that contains it and DNA processing mechanisms.

1.6. Goals of the project

The objective of this project was to reveal novel proteins containing UBZ4 domain and to characterize ubiquitin-binding features of selected ones. From the literature it is known that UBZ4 domain is a zinc-finger like ubiquitin-binding domain but only few proteins containing them were investigated (Wrnip1 and Rad18). Experiments so far showed that UBZ4 as a domain is mainly engaged in DNA processing so it would be interesting to find new proteins containing this domain.

To accomplish this I performed iterative alignment using isolated sequence of UBZ4 from Wrnip1 to get a number of proteins with putative UBZ4 domain.

Protein of interest (C1orf124) (accession number AAH68478) was then analysed using standard biochemical techniques.

I was mainly interested in the nature of protein binding to Ub. I wanted to elucidate if the putative UBZ4 domain on the C-terminal end of protein was responsible for its binding to different Ub species. I also wanted to reveal if UBZ4 domain recognizes a hydrophobic surface patch surrounding the highly conserved Ile-44 residue of Ub and which amino-acid inside the domain is responsible for this interaction.

2. Materials and methods

2.1. Materials

The following items were purchased from Amersham Biosciences (GE Healthcare Life Sciences) (England):

- Anti-Mouse IgG

The following items were purchased from AppliChem Co. (Germany):

- 40% acrylamide / 0,8% bisacrylamide solution
- Glycerol
- Sodium dodecylsulphate (SDS)
- Tris base and Tris-HCl

The following items were purchased from BD Biosciences-Clontech (USA):

- Difco agar

The following items were purchased from BIO-Rad Co. (USA)

- All the equipment to run SDS-PAGE
- Precision plus protein™ Dual color standards

The following items were purchased from Fermentas International INC. (Canada)

- BamHI restriction enzyme with its buffer
- EcoRI restriction enzyme with its buffer
- NotI restriction enzyme with its buffer
- SalI restriction enzyme with its buffer

The following items were purchased from GIBCO Co. (USA):

- Ultra pure water (H₂O)
- Penicillin-streptomycin

The following items were purchased from Invitrogen Co. (USA):

- Agarose
- Chemically competent *Escherichia coli* of DH5α strain
- Lipofectamine reagent

The following items were purchased from New England Biolabs Inc. (USA)

- 1kb DNA Ladder
- DpnI restriction enzyme and its buffer
- T4 ligase and its buffer

The following items were purchased from Qiagen Co. (Germany)

- QIAEX II Gel Extraction kit

- QIAprep Spin Miniprep kit

The following items were purchased from ROTH Co. (Germany)

- Ampicillin
- B-mercaptoethanol
- Bovine Serum Albumine (BSA)
- Bromphenol Blue (BPB)
- Ethidium bromide (EtBr)
- Ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA)
- Milk powder
- Phenylmethanesulphonylfluoride (PMSF)
- Ponceau S
- Sodium acetate
- N,N,N,N-tetramethylethylenediamine (TEMED)
- Triton X-100
- All inorganic salts and solvents

The following items were purchased from Roche Co (Switzerland):

- Deoxynucleoside triphosphates (dNTPs)

The following items were purchased from Sigma-Aldrich (USA):

- Anti-FLAG M5 monoclonal antibody
- Ammonium persulphate (APS)
- Aprotinin
- Dimethyl sulfoxide (DMSO)
- Dulbecco's Modified Eagle's Medium (DMEM)
- Hydroxyethyl-piperazineethanesulphonic acid (HEPES)
- Leupeptin hemisulfate
- Sodium orthovanadate

Other items were purchased from the following sources:

- DNA Polymerase Pfu Ultra High Fidelity (with buffer) from STRATAGENE Co. (USA)
- Enhanced chemiluminescence reagents from Santa Cruz Biotechnology (USA)
- Ethylenediaminetetraacetic acid disodium salt-2-hydrate (Na_2EDTA) from Riedel-deHaen Co. (Germany)
- Fetal Bovine Serum from PAA Co. (Austria)
- Human embryonic kidney (HEK) 293 cells from ATCC Co. (USA)

- Nitrocellulose membranes from Osmonics Co. (USA)
- Whatman filter papers from Whatman Co.

2.1.1. Constructs

- pCMV-FLAG-C1orf124 construct was prepared as described in methods
- pCMV-FLAG-C1orf124 (D479A) construct was generated by site-directed mutagenesis of residue Asp479 to alanine
- pGST-UBZ construct was prepared as described in methods
- pGST-Ub construct was prepared in Ivan Đikić's lab
- pGST-tetraUb construct was prepared in Ivan Đikić's lab
- pGST-Ub (I44A) construct was prepared in Ivan Đikić's lab

All constructs listed above are resistant to Ampicillin.

2.1.2. Antibodies

- anti-FLAG M2 monoclonal antibody from Sigma Aldrich (USA)

2.1.3. Buffers

50x TAE buffer

Tris	2 M
EDTA	0,5 M
Acetic acid	1 M

10x DNA loading buffer

Glycerol	50%
EDTA	0,1 M
SDS	1%
Bromophenol blue	0,2%
Xylene cyanol	0,2%

GST-Buffer 1

Tris-HCl, pH 7,5	0,02 M
EDTA, pH8	0,01 M
EGTA	5 mM
NaCl	0,15 M
PMSF	1 mM
βMe	0,1%

GST-Buffer 2

Tris-HCl	0,02 M
EDTA	0,01 M
NaCl	0,15 M
Triton X-100	0,005%
PMSF	1 mM
βMe	0,1%

GST-Buffer 3

Tris-HCl	0,02 M
βMe	0,1%
NaN ₃	0,1%

Lysis Buffer, pH 7,5

HEPES	50 mM
NaCl	150 mM
EDTA	1 mM
EGTA	1mM
NaF	25 mM
Triton X-100	1%
Glycerol	10%
ZnCl ₂	10 μM

Protease inhibitors:

PMSF	1mM
Leupeptin	2 μg/ml

Aprotinin	10 µg/ml
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Phosphatase inhibitor:

Sodium Orthovanadate	1mM
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Separating gel buffer

Tris-HCl, pH 8,8	1,5 M
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SDS	0,4%
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Stacking gel buffer

Tris-HCl, pH 6,8	0,5 M
------------------	-------

SDS	0,4%
-----	------

TE buffer

Tris	10 mM
------	-------

Na ₂ EDTA	1 mM
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10x thrombine cleavage buffer

TrisCl, pH 8,4	200mM
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NaCl	1.5 M
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CaCl ₂	25 mM
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2 x Leammli-Sample Buffer

Tris, pH 6,8	25 ml
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Glycerol	20 ml
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SDS, 10%	20 ml
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Bromophenol blue	1 mg
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β-merkaptoethanol	5 mL
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10 x PBS (Phosphate-Buffered Saline), pH 7,3

NaCl	80 g
------	------

KCl	2 g
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NaH ₂ PO ₄ *7H ₂ O	11,5 g
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KH ₂ PO ₄	2 g
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10 x Running buffer

Tris	30 g
Glycine	144 g
SDS	10 g
dH ₂ O	up to 1 l

10 x Transfer buffer

Tris	22,3 g
Glycine	105 g
dH ₂ O	up to 1 l

20 x TBS (Tris-Buffered Saline), pH 7,6

Tris	201,17 mM
NaCl	1,198 M

2.1.4. Solutions and plates

Ponceau S solution

Ponceau S	0,5 g
Acetic acid	10 ml
dH ₂ O	up to 100 ml

Western blot blocking solution, pH 7,5

1 x TBS

BSA	5%
Na-azide	0,1%

1% BSA (Bovine Serum Solution)/PBS solution

BSA was dissolved in PBS buffer and pH was adjusted to 7,5

The media (both liquid and solid) were autoclaved at 121°C, 15 min.

LB medium and plates

Bacto tryptone	10 g
Bacto yeast extract	5 g

NaCl	5 g
Difco agar (for plates only)	15 g
dH ₂ O	up to 1 l
Ampicillin	100 µg/ml (added after autoclaving, after the solution had cooled to ≈55°C)

2.1.5. Gels

1.5% agarose gel

Agarose	3.75 g
1 x TAE Buffer	250 ml

Solutions were boiled using microwave to dissolve.

After running, gels were kept 30 min in 1:100000 ethidium bromide solution for staining.

Polyacrylamide gels

Table 2. Recipes for polyacrylamide separating and stacking gels

<i>Stock solution (3 gels)</i>	Separating (lower) gel		Stacking (upper) gel
Final acrylamide concentration	7%	8%	3,9%
Lower buffer / (ml)	3,750	3,750	-
Upper buffer / (ml)	-	-	1,25
ddH ₂ O / (ml)	8,625	8,250	3,21
10% APS / (µl)	50	50	25
TEMED / (µl)	10	10	5
40% acrylamide/0,8% bisacrylamide / (ml)	2,625	3,000	0,49

2.2. Methods

2.2.1. Bioinformatical tools

PSI-BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to perform multiple alignment of the Wrnip1 UBZ domain sequence with the E-value equal 10^{-3} and three-iterations against the NCBI nonredundant protein sequence database (nr database) (Jones and Swindell, 2002; Altschul et al. 1997; Altschul et al. 1998)

Obtained sequences were then aligned using CLUSTALW2 multiple alignment tool (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) (Chenna et al. 2003).

Sequence of C1orf124 protein was analysed in Pfam database (<http://pfam.sanger.ac.uk/>) (Finn et al. 2006)

2.2.2. Molecular cloning

2.2.2.1. Amplification

The following plasmidic constructs were prepared and used for the experiments here described: pCMV FLAG – C1orf124 using NotI and SalI and pGEX – UBZ (C1orf124) using EcoRI and BamHI. Primers used for cloning of these constructs are listed in the Table.

Table 3. List of primers and their sequences used for molecular cloning

Name of the primer	Sequence
ORF-FLAG for	5' ATTGCGGCCGCCGATGATGACTTGATGTTG 3'
ORF-FLAG bac	5' GCGGTCGACTCAAAGACTTTCTTCGCTTTT 3'
ORF-GEX for	5' CGCGGATCCAAAATGGTTAATTGCCCA 3'
ORF-GEX bac	5' GCCGAATTCGTATTTGATAGTGTCACC 3'

The template cDNA used in the generation of Wrnip1 constructs was obtained from the German genomic consortium RZPD (Item No. IRATp970E1156D – Full length clone). Primers were obtained from MWG BIOTECH, Martinsried.

Table 4. PCR reaction mix

DNA template	20 ng
10x pfu DNA polymerase Buffer	5 µL
Primer-forward (10 µM)	1 µL
Primer-backward (10 µM)	1 µL
dNTPs	1 µL
Pfu DNA polymerase (2,5 U/µL)	1 µL
ddH ₂ O	up to 50 µL

PCR program used is indicated in table below

Table 5. PCR program for amplification of cDNA

Segment	Cycles	Temperature	Time
1	1	95°C, denaturation	10 min
2	16	95°C, denaturation	30 s
		62°C, annealing	30 s
		72°C, elongation	1 min
3	1	72°C, final elongation	10 min
4	1	4°C	∞

2.2.2.2. Restriction digestion

Restriction digestion of the amplification products

Table 6. Reaction mixes for restriction digestion

	pCMV FLAG C1orf124	pGEX UBZ C1orf124	pCMV-FLAG	pGEX 2T
DNA	20 µL	20 µL	10 µL	10 µL
10x Buffer O	5 µL	-	5 µL	0 µL
10x Buffer Tango	-	10 µL	-	10 µL
EcoRI restriction enzyme (2,5U/µL)	-	1 µL	-	1 µL
BamHI restriction enzyme (2,5U/µL)	-	1 µL	-	1 µL
NotI restriction enzyme (2,5U/µL)	1 µL	-	1 µL	-
Sall restriction enzyme (2,5U/µL)	1 µL	-	1 µL	-
ddH ₂ O	up to 50 µL	up to 50 µL	up to 50 µL	up to 50 µL

Restriction digestion was performed on 37°C for 4 hours.

Gel extraction of the inserts

Inserts were separated from the template by electrophoresis on 2% agarose gel. Inserts were cut out of the gel and extracted from it with QIAEX II Gel extraction kit according to provided protocol.

2.2.2.3. Ligation

Table 7. Ligation mix

10x T4 DNA Ligase Buffer	1 µL
Vector	4,5 µL
DNA insert	4,5 µL
T4 DNA ligase (10U/µL)	0,5 µL

Ligation was performed on 16 °C over night.

2.2.2.4. DH5α transformation

For bacterial transformation with competent cells 5 µL of the ligation mix and 50 µL of DH5α competent cells were taken. The mixture was gently mixed and kept on ice for 30 minutes. After incubation period, bacteria were heat-shocked in termoblock for 45 seconds on 42°C and afterwards put on ice for 2 minutes. The whole mixture was transferred to 1 mL pre-heated LB medium and shaken for 1 hour on 37°C. Thereafter, the mixture was centrifuged (5 min, RT, 5 000 x g) and 900 µL of supernatant was discarded. The pellet was resuspended in the remaining media and plated on LB plates containing Ampicillin. The plates were incubated overnight on 37°C.

2.2.2.5. Plasmid amplification

Single bacterial colonies were picked up from overnight plates. Colonies were put into 5 µL LB medium containing Ampicillin and left overnight at 37°C. Next day QIAprep Spin Miniprep kit was used to purify plasmid according to manufacturer's protocol.

2.2.2.6. Sequencing

DNA concentration was detected using spectrophotometer (Eppendorf-Biophotometer). One µg of plasmid DNA was lyophilized and sequenced by MWG BIOTECH, Martinsried (<https://ecom.mwgdna.com/services/home.tc>) using commercially available primer. The DNA sequence identification was performed using BLAST (www.ncbi.nlm.nih.gov/BLAST).

2.2.3. Site directed *in vitro* mutagenesis

2.2.3.1. Amplification

Table 8. PCR reaction mix used for site directed *in vitro* mutagenesis

DNA template	20 ng
10x Pfu DNA polymerase buffer	5 µL
Primer-forward (10 µM)	1 µL
Primer-reverse (10 µM)	1 µL
dNTPs	1 µL
Pfu DNA polymerase (2.5 U/µL)	1 µL
ddH ₂ O	Up to final volume of 50 µL

PCR program used for site directed mutagenesis is indicated in table below

Table 9. PCR program used for site directed *in vitro* mutagenesis

Segment	Cycles	Temperature	Time
1	1	95°C, denaturation	1 min
2	18	95°C, denaturation	1 min
		55°C, annealing	1 min
		68°C, elongation	16 min
3	1	68°C, final elongation	16 min
4		4°C	∞

Table 10. List of primers used for site directed *in vitro* mutagenesis

Primers	Template	Purpose
Forward: CAGATTAATGAGCACTTGGCCTGGTGCCTTGAA GGTGAC Reverse: GTCACCTTCAAGGCACCAGGCCAAGTGCTCATT AATCTG	pCMV FLAG – C1orf124	pCMV FLAG – C1orf124 D479A

2.2.3.2. DpnI treatment

Dpn I digestion of the amplification products

PCR product 50 μ L

Dpn I restriction enzyme (10 U/ μ L) 1 μ L

1 hour, 37°C incubation

The basic procedure utilizes a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by PfuTurbo DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to Dpn I digestion. The mixture was used for DH5 α bacterial transformation.

2.2.3.3. DH5 α transformation

DH5 α transformation was performed as described above with 5 μ L of Dpn I digestion product and 100 μ L of competent bacteria cells.

2.2.3.4. Plasmid amplification

Plasmid amplification was performed as described above.

2.2.3.5. Sequencing

Sequencing was performed as described above.

2.2.4. GST protein purification

2.2.4.1. *BL21 transformation*

For bacterial transformation with competent cells 500 ng of plasmid and 50 μ L of BL21 competent cells were taken. Transformation was performed as described above.

2.2.4.2. *Protein expression induction and purification*

Single colonies were picked up from over night plates. Colonies were put into 5 mL LB medium containing Ampicillin and left to shake over night at 37°C. The day after, 5 mL culture was added to 200 mL of fresh LB medium containing ampicillin and put to shake at 37°C until optical density of culture reached 0,35 – 0,6 when 0,5 mM IPTG was added. Culture was put to shake at 37°C for 4 hours. Bacteria were centrifuged (20 min, 4°C, 5000 x g) and supernatant discarded. Pellet was resuspended in 40 mL of chilled PBS in Falcon tube. Bacteria were again centrifuged (20 min, 4°C, 5000 x g) and supernatant discarded. Pellet was resuspended in 20 mL of GST Buffer 1. Suspension of bacteria cell was kept on ice and sonicated 4 times for 1 minute with 1 minute of cooling down interval in between. After sonication 500 μ L of 20% Triton X-100 was added to suspension. Suspension was transferred to centrifuge tubes and centrifuged (20 min, 4°C, 10 000 x g). Glutathione sepharose beads were washed 3 times in 500 μ L and added to supernatant. Suspension was then incubated on rotator over night at 4°C. Next day beads were washed 3 times in 30 mL of GST Buffer 2 and resuspended in 2 mL of GST Buffer 3.

Different amounts of GST fusion protein suspension were mixed with Laemmli buffer boiled on 95°C and ran on SDS-PAGE. The gel was stained with Coomassie blue solution and destained with de-staining solution. The amount of GST fusion protein was determined.

2.2.4.3. *Preparation of Ub, I44A-Ub and 4xUb proteins*

200 μ L of GST-Ub, GST I44A-Ub and GST 4xUb bound to Glutathione beads were taken for further preparation. Beads were washed three times in 600 μ L of thrombin cleavage buffer and added to 200 μ L of cleavage buffer containing 2 U of thrombin. Mixture was incubated over night on 22°C. After incubation PMSF was added to

inhibit thrombine. Mixture was spun down and supernatant was used for GST pull down assay or stored at 4°C

2.2.5. Cell culture

HEK293T cells were grown and maintained in a cell tissue incubator, in 5% CO₂ humid atmosphere at 37°C and cultured in DMEM supplemented with 10% fetal bovine serum. Appropriate antibiotics were added to growth media; penicillin (100 U/mL) and streptomycin (100 µL/g). Passaging was performed every 2-3 days.

2.2.6. Transfections

Transient transfections were performed for overexpression experiments. Agent for transfection was Lipofectamine 2000 reagent. One day before transfections cells were plated according to manufacturer's protocol (the amount of cells is in proportion to the relative surface area belonging to culture vessel)

Cells were transfected using DNA (µg) to Lipofectamine 2000 reagent (µL) ratio of 1:3. For 6-well dishes, 0.5 µg of DNA were used. According to manufacturer's protocol Lipofectamine 2000 reagent was mixed with serum free medium (DMEM medium without serum) and incubated for 5 minutes. DNA was diluted in serum free media and mixed gently. After 5 minutes incubation, diluted DNA was combined with diluted Lipofectamine 2000 reagent. Mixture was mixed gently and incubated for 30 minutes at room temperature. Before complex was added to cells, fresh serum free medium was added to cells. After incubation period complexes were added to cells and dishes are mixed gently by rocking the plate. Transfections were stopped 4-6 hours after, by changing serum free medium to medium with serum.

Plasmids used for transfection were pCMV FLAG – C1orf124 wt and pCMV FLAG – C1orf124 D479A

2.2.7. Preparation of cell lysates

Thirty-six hours after transfection cells were lysed. Dishes containing cells were put on ice, the media was removed by suction and cells were washed with 500 μ L of cold PBS buffer (-Ca, -Mg). After washing, 400 μ L of ice cold lysis buffer was added to each well of 6-well plate. After 30 minute incubation on ice, cell lysates were scraped, transferred to pre-cooled 1.5 mL tube and clarified by centrifugation (25 min, 4°C, 16 000 x g) to remove Triton X-100 insoluble fraction. TCL (Total Cell Lysate) and Leammli buffer were mixed in 1:1 ratio, boiled for 5 minutes on 95°C to prepare samples for performing Western Blotting. The remaining TCLs were used for GST-pull down assays. If the lysates were not used immediately after preparation, they were stored at -20°C.

2.2.8. GST pull down assay

GST pull down assay was performed using following GST fusion proteins bound on Glutathione sepharose beads : GST-empty, GST-Ub, GST-4xUb, GST-I44A Ub, GST-UBZ (C1orf124); TCLs: FLAG C1orf124 and FLAG C1orf124 D479A; thrombine cleaved proteins: Ub, I44A Ub, 4XUb.

Following mixtures were prepared:

Mix 1

GST-empty	5 μ L
TCL – FLAG-C1orf124	100 μ L
Lysis buffer	500 μ L

Mix 2

GST-Ub	7 μ L
TCL – FLAG-C1orf124	100 μ L
Lysis buffer	500 μ L

Mix 3

GST-4xUb	7 μ L
TCL – FLAG-C1orf124	100 μ L

Lysis buffer	500 µL
Mix 4	
GST-I44A Ub	7 µL
TCL – FLAG-C1orf124	100 µL
Lysis buffer	500 µL
Mix 5	
GST-empty	5 µL
TCL – FLAG-C1orf124 D479A	100 µL
Lysis buffer	500 µL
Mix 6	
GST-Ub	7 µL
TCL – FLAG-C1orf124 D479A	100 µL
Lysis buffer	500 µL
Mix 7	
GST-4xUb	7 µL
TCL – FLAG-C1orf124 D479A	100 µL
Lysis buffer	500 µL
Mix 8	
GST-I44A Ub	7 µL
TCL – FLAG-C1orf124 D479A	100 µL
Lysis buffer	500 µL
Mix 9	
GST-UBZ (C1orf124)	10 µL
Thrombine cleaved Ub	20 µL
Lysis buffer	500 µL
Mix 10	
GST-UBZ (C1orf124)	10 µL
Thrombine cleaved I44A Ub	20 µL

Lysis buffer 500 μ L

Mix 11

GST-UBZ (C1orf124) 10 μ L

Thrombine cleaved 4xUb 20 μ L

Lysis buffer 500 μ L

Mixtures were incubated on a rotator at 4°C over night. After incubation beads were washed three times with 600 μ L of lysis buffer. After last washing beads were spun down, supernatant was discarded and 40 μ L of Leammli sample buffer was added. Samples were heated at 95°C for 5 minutes.

2.2.9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Samples were prepared as described previously, then separated by SDS-PAGE (80 V for upper gel and 120 V for lower gel) and transferred to nitrocellulose membrane (200 mA, 60 min) in 1 x transfer buffer, supplemented with 20% (v/v) methanol. The total level of loaded and transferred proteins was revealed by incubating membrane in Ponceau S solution for 1 min and afterwards shortly washed in dH₂O. The staining is reversible, so the membrane was then washed in TBS buffer, two times for 5 min, and blocked in TBS containing 5% BSA for 1.5 hours at RT. Immunoblotting was performed overnight with the anti-FLAG M2 monoclonal primary antibodies diluted in TBS with 5% BSA at +4°C in 1:10 000 ratio. After overnight incubation, membrane was washed three times in TBS containing 0.05% Triton X-100 for 10 min each, the membrane was incubated with secondary antibody (anti-mouse-IgG), for 1 hour at RT. Secondary antibody was prepared in filtered TBS with 5% milk powder and 0.05% Tween. After incubation, membrane was subsequently washed three times as before. Proteins levels were finally revealed using enhanced chemiluminescent reagents according to the manufacturer's instructions. If needed, membranes were afterwards striped in 0.2 NaOH solution for 5 minutes.

3. Results

3.1. C1orf124 protein contains putative UBZ domain

PSI-BLAST analysis of Wrip1 UBZ domain sequence gave set of 13 proteins which showed evolutionary relationship with input domain. All of 13 proteins in the set contained UBZ4 domain characteristics. Multiple alignment of obtained protein set showed that CCHC UBZ4 motif is highly conserved as well as aspartate residue in-between second Zn-binding dyad.

WRNIP1	hs	HQVQCPVCQQMMPAAH...INSHLDRCLLLHPAG
MGS1	sc	QLISCPICSRKVFFSL...INSHLIDICGKEKSKP
RAD18	hs	TKVDCPVCGVNIPESH...INKHLIDSCLTSREEKK
RAD18	sc	QMAQCPICQQFYPLKA...LEKTHLIDECLTLQSLG
DSLRE1A	hs	YDGYCPNCQMPFSSLIGQTPRWVFECLDSPPRS
PSO2	sc	EVIQCPICLENLSHLELYERETHCDTCTIGSDPSH
POLK	hs	QILTCPVCFRAQGCISLEALNKHVDECLDGPSIS
		QALVCPVCNVEQKTSDLTLENVHVDVCLNKSFIQ
ZNRF1	hs	SGFKCPICSKSVASDE...MEMHFIMCLSKPRLS
ZNRF2	hs	GGFKCPVCSKFVSSDE...MDLHLVMCLTKPRIT
UIMC1	hs	NQVSCPLCDQCFPTK...IERHAMYCNGIMEED
UIMC1		KNEKCYLCKSLVPFRE...YQCHVDSCLQLAKAD
C1orf124	hs	KMVNCPVCQNEVLESQ...INEHLIDWCLEGDSIK
C6orf94	hs	ELFPCEVCGRRFAADV...LERHGPICKKLFNRK
		DYIQREYCMRRFNESA...AERHTNECKDQSSRR
C8orf70	hs	ELLPCIKCGRTFFPVA...LKKHGPICKQTATKK
		DYIQCEYCORRFNENA...ADRHINECKEQAARI

Figure 8. Multiple alignment of protein set obtained by PSI-BLAST.
Zn-binding motif is presented in red. Highly conserved aminoacids are presented in black.

Novel proteins from data set were analyzed in Pfam protein family database and protein C1orf124 was chosen for further experiments. Pfam database analysis of C1orf124 protein showed presence of the zinc binding region inside SprT-like metalloprotease domain starting at 41st and ending at 207th amino acid residue.

3.2. C1orf124 is binding to monoubiquitin and polyubiquitin chains

In order to confirm binding of C1orf124 to different ubiquitin species GST pull down assay was performed. As a stationary phase GST-constructs of ubiquitin species (GST-Ub – with only one ubiquitin, GST-4xUb, GST-Ub I44A mutated in hydrophobic patch region) were used and as a mobile phase total cell lysate of HEK293T cells overexpressing FLAG-tagged C1orf124 protein.

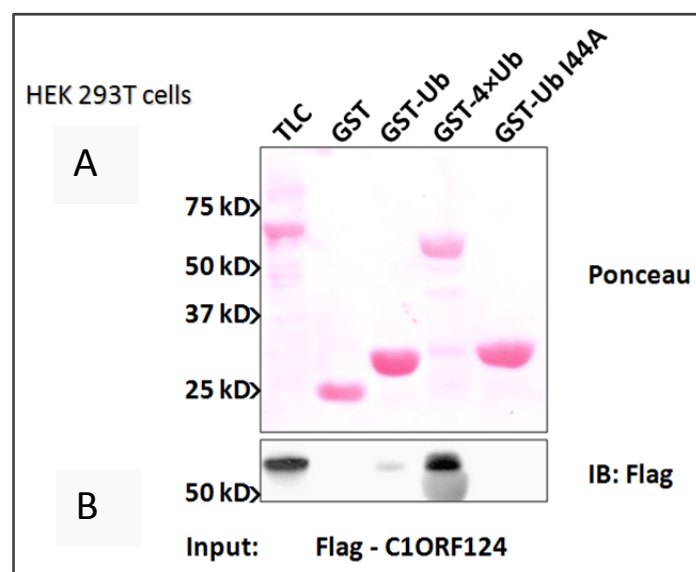


Figure 9. Binding of C1orf124 protein to different ubiquitin species. A: Protein blot on membrane stained with Ponceau; B: Western blotting on proteins pulled down by GST-Ub and GST-4xUb detected with antibody against FLAG tag.

Figure 9A presents Ponceau stained membrane, and 9B results of Western blotting of pulled down proteins. Western blotting was performed with antibody against FLAG tag (lane 1, MW=60 kD). These experiments showed the presence of the overexpressed protein C1orf124 in TCL. Pull down assay revealed different affinity of C1orf124 for different substrates: our protein was bound strongly to Ub chains containing 4 Ub moieties (lane 4B) and binding weakly to monoUb (lane 3). Protein didn't bind to empty GST protein which served as a negative control (lane 2). It also didn't bind to GST-Ub I44A construct which contains mutation inside hydrophobic patch, indicating the role of this hydrophobic patch for UBZ 4 binding.

3.3. UBZ4 domain of C1orf124 protein is responsible for binding to different Ub species

We wanted to show whether UBZ4 domain C1orf124 was responsible for ubiquitin binding so we used GST constructs containing isolated UBZ domain C1orf124 protein. These constructs were used as a stationary phase and as a mobile phase purified Ub, UbI44A and 4xUb. Western Blotting assay was done afterwards.

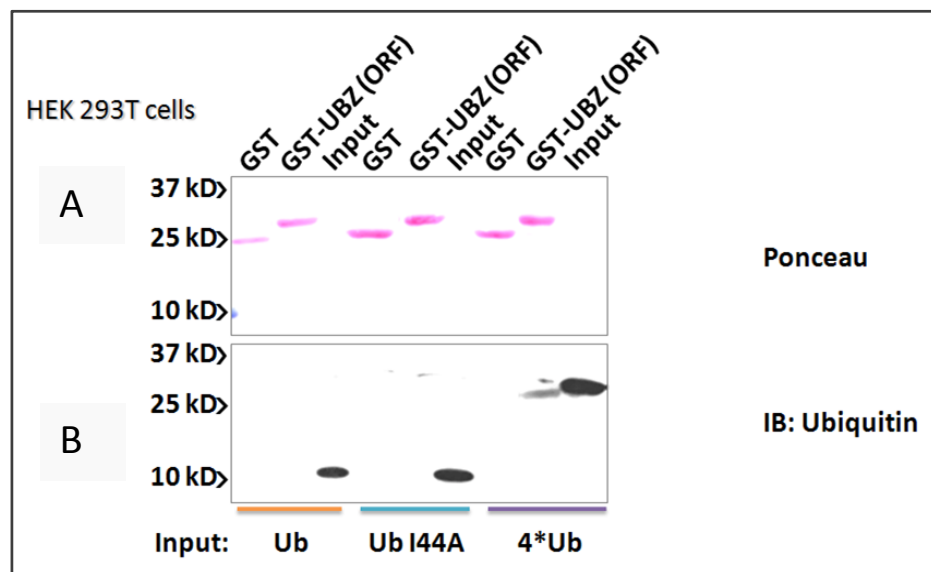


Figure 10. UBZ4 domain of C1orf124 protein is responsible for binding to Ub.

A: Protein blot on membrane stained with Ponceau; B: Western blotting on ubiquitin species pulled down by GST-UBZ4 detected with antibody against ubiquitin.

Figure 10 shows the results: Figure 10A presents Ponceau stained membrane and figure 10B Western blotting on ubiquitin species pulled down with isolated UBZ4 in GST construct. Blotting with anti-Ub antibody showed that UBZ4 domain of C1orf124 antibody is strongly bound to 4xUb (lane 8), while binding to mono Ub (lane 2) and mutated UbI44A (lane 5) was abolished. These results indicate specificity of C1orf124 binding to Ub.

3.4. Aspartate residue in-between second Zn-binding dyad of UBZ domain is responsible for binding of C1orf124 protein to Ub species

To further investigate the binding properties of our protein, we performed reversed pull down assay. Mutant of C1orf124 was prepared with aspartate residue in-between second Zn-binding dyad of UBZ mutated into alanine residue. This construct was examined on its ability to pull down different Ub species. GST pull down assay was performed with GST constructs of different Ub species as a stationary phase and TCL of HEK293T cells overexpressing FLAG-tagged mutant C1orf124 protein as a mobile phase, followed by Western Blotting.

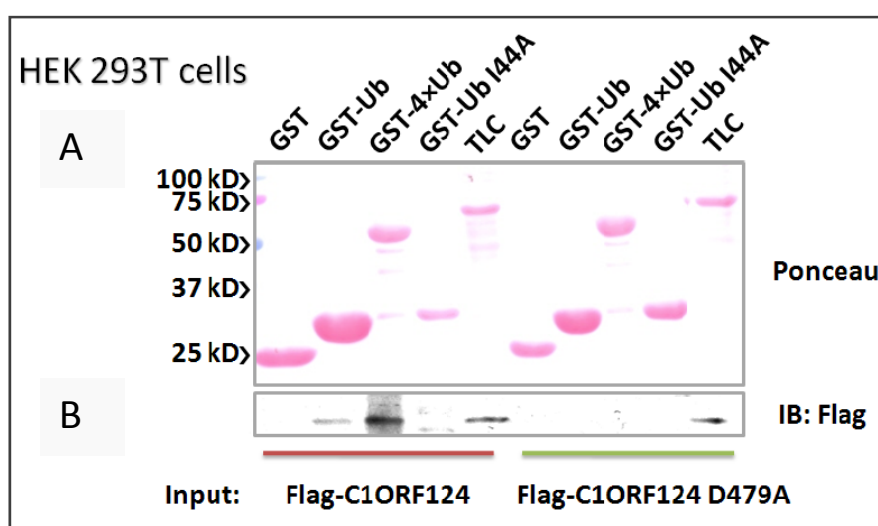


Figure 11. Mutant C1orf124 protein showed no binding to any Ub specie.

A: Protein blot on membrane stained with Ponceau; B: Western blotting on proteins pulled down by GST-Ub and GST-4xUb detected with antibody against FLAG tag

Figure 11B shows the results of Western blotting of proteins detected with antibody against FLAG tag. Presence of protein C1orf124, with mutated aspartate residue into alanine residue in the total cell lysate confirmed expression in the cell (lane 5) but binding to all Ub species constructs, GST-Ub (lane 2), GST-4xUb (lane 3) and GST-UbI44A (lane 4) was abolished.

These results confirmed the key role of Asp497 in the UBZ4 domain for the ubiquitin binding.

4. Discussion

PSI-BLAST analysis provided us with 13 protein member dataset containing putative UBZ4 domains. Proteins from the dataset showed sequence features that corresponded with UBZ4. We could see two highly conserved Zn-binding dyads (C2 and HC). They are important for this protein family as they bind Zn atom coordinately and provide domain with specific structure (Figure 8.). Aspartate residue in-between of second Zn-binding dyad is also highly conserved and crucial for binding as I showed. From this 13 member protein set we picked the C1orf124 protein for further analysis.

Pfam database analysis of protein C1orf124 indicated presence of SprT-like metalloprotease domain starting at 41st and ending at 207th amino acid residue. SprT-like metalloprotease family represents approximately 160 residues in a group of proteins conserved from fungi to humans. It is still uncharacterised. Presence of the protease domain and ubiquitin-binding domain in the same protein could easily indicate deubiquitinating protease function.

In order to experimentally analyze the UBZ4 domain and its binding substrates, we cloned C1orf124, expressed it in several animal cell lines and performed pull down assay with different Ub species.

Our results showed that protein C1orf124 bound with high affinity to 4xUb, and with lower to mono Ub. 4xUb is the smallest fragment of polyUb chain recognised by domains that bind to Ub polymers. Probably UBZ4 protein can recognise hydrophobic patch around Ile44 on single Ub moiety but the overall affinity was lower.

To specify the binding domain of the protein I made a GST chimera containing UBZ4 domain of protein C1orf124. Isolated domain showed binding to polyUb chains but not to monoUb or to Ub I44A mutant (mutant with Ile to Ala; responsible for Ub binding) (De Fiore *et al.* 2003). Ub itself is a small protein usually recognised bound to its targeted molecule, so these results suggest that the possibility of interaction between full length Ub-binding protein and ubiquitylated protein.

For further characterization of binding domain, we used C1orf124 mutant (mutant with Asp to Ala in-between second Zn-binding dyad of UBZ4; responsible for recognition of Ub). This mutant did not bind to any of the Ub species. This could indicate the crucial role of Asp in Ub binding. Analogous to UBZ3 structure (Figure 7.) Asp479 residue could be on the opposite side of α -helix in the domain and the formation of Zn-finger structure actually could push it outside on to find its interactor. The only UBZ4 domain protein analyzed in details up to now is Wrnip1. Its function is still elusive. Characterization of its UBZ4 domain showed ability to bind mono Ub

and poly Ub as well as increasing presence and localisation in DNA replication sites during DNA damage mediated by UBZ4 domain. In comparison, C1orf124

In comparison, the novel protein C1orf124 containing very interesting combination of domains could be one of the nuclear proteins localising in DNA replication factories.

If it's known that UBZ4 domain is often found in proteins that are involved in DNA repair processes then in combination with protease domain it can suggest a function of regulation of ubiquitinated proteins in DNA replication factories or nucleus it self.

5. Conclusion

In this project we found bioinformatically 13 potential protein containing UBZ4 domain.

We cloned one of these proteins, C1orf124 and characterized its UBZ4 domain.

We analysed binding properties of C1orf124 containing UBZ4 domain. Cloned C1orf124 showed strong affinity toward polyUb, weak affinity toward monoUb and did not bind mutated Ubi44A with abrogated key aminoacid.

Cloned UBZ4 domain showed strong affinity to polyUb but didn't bind to monoUb or mutated one.

Reverse experiments showed abrogation of Ub binding to C1orf124 mutated in Asp located in-between second Zn-binding dyad indicating the key role of this residue in these interactions.

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